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Organochlorine Pesticide Levels in Maternal Serum and Risk of Neural Tube Defects in Offspring in Shanxi Province, China: A Case-Control Study

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Abstract

Organochlorine pesticides (OCPs) in placental tissue have been reported to be associated with an increased risk for fetal neural tube defects (NTDs). Our case-control study was performed to explore the association between maternal serum OCP concentration and NTD risk in offspring. Serum samples were collected from 117 mothers who delivered NTD infants (case group) and 121 mothers who delivered healthy infants (control group). Only three of the 25 OCPs were detected in more than half of the maternal serum samples. The median concentration of total OCPs in the case group was significantly higher than that of the control group. However, no dose-response relationships between higher levels of any individual OCP or total OCPs and the risk of NTDs or subtypes were observed in either the unadjusted binary unconditional logistic regression model or the model adjusted by potential confounders. We conclude that no clear association between maternal serum OCP residues and NTD risk in offspring was observed in this population.

Keywords

neural tube defect; risk; organochlorine pesticides; maternal serum; case-control study

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1. Introduction

Neural tube defects (NTDs) are a group of congenital malformations resulting from the failure of the neural tube to close by the 28th day of conception. NTD rate is about one in every 1000 established pregnancies worldwide, ranging from 0.2 to 10 per 1000 depending on the specific geographical locations (Copp et al., 2013). In developed countries, like in United States, NTD rate is 0.5 in 1000 births, while it is considerably higher in developing areas such as Latin America (2.2 per 1000 births in Argentina and 3.1 per 1000 births in Brazil) (Castilla et al., 2003) and China (13.9 per 1000 births) (Li et al., 2006). NTDs result in both great economic and psychological burdens for the patients and their family. Thus, NTDs continue to be a major public health issue and are gaining extensive attention around the world (Wallingford et al., 2013).

The etiology of NTDs is very complex. Folate acid supplementation has been proved to be an efficient way to reduce the risk of fetal NTDs by both observational studies and randomized trials (Copp et al., 2013), but the underlying mechanism of NTD prevention with folic acid remains poorly understood (Wallingford et al., 2013). On the other hand, 30–50% of NTD cases are not folate preventable, and other factors must be considered (Blom et al., 2006). Environmental pollutants are implicated in the development of NTDs from both animal experiments (Barbieri et al., 1986) and a human epidemiological study on occupational exposure to polycyclic aromatic hydrocarbons (Langlois et al., 2012).

Organochlorine pesticides (OCPs) were extensively used in agriculture throughout the world between 1950 and 1970 (Li and Macdonald, 2005). Most of OCPs were banned for use in 1970s in many countries, but many developing countries continue using them in agricultural industry and for malaria control due to their low-cost and effectiveness. Although OCPs production and application in China were officially prohibited in 1983 (Nakata et al., 2002), they have been detected in soil (Zhou et al., 2013), food (Yu et al., 2012), maternal blood (Qu et al., 2010), and even umbilical cord blood (Yu et al., 2013) and placenta (Ren et al., 2011) in recent years. OCPs have attracted worldwide concern for decades because of their long half-lives and potential adverse effects on both environment (Nakata et al., 2002) and human health (Androutsopoulos et al., 2013).

Despite their presence at low levels in humans, they can have adverse effects by interacting with a wide range of enzymes, proteins, receptors and transcription factors, resulting in reproductive and developmental defects (Androutsopoulos et al., 2013). For example, *p,p'*-dichlorodiphenyldichloroethylene (*p,p'*-DDE) at high concentrations can act as an inhibitor of 5 α -reductase, the enzyme that converts testosterone to dihydrotestosterone (Frye et al., 2012). Long-term environmental exposure to certain OCPs (i.e., lindane and dieldrin) has been associated with the development of neurodegenerative disorders (Heusinkveld and Westerink, 2012).

Two previous studies found that maternal exposure to OCPs could potentially increase NTD risk in the offspring (Brender et al., 2010; Shaw et al., 1999). Placental samples were used to investigate the association between placental levels of OCPs and the NTD risk in our previous study, and a dose-response relationship between OCPs (including *o,p'*-

dichlorodiphenyltrichloroethane and metabolites, and hexachlorocyclohexanes) and NTD risks was observed (Ren et al., 2011). OCP residues bound to fat in maternal body can be released into blood stream (Noren et al., 1999), and then transferred to fetus through the placenta (Longnecker et al., 1999). Levels of polychlorinated biphenyls, *p,p'*-DDE, and hexachlorobenzene in the placenta were found to correlate well with those measured in maternal blood serum (Bergonzi et al., 2009; Yu et al., 2013). Therefore, the maternal OCP concentrations could present the exposure of fetus. However, human blood sample is not always available in sufficient amounts for a reliable analysis for OCPs compared with other biospecimens, such as human hair, and its reliability is still in dispute (Michalakis et al., 2013; Tsatsakis et al., 2008). Even though these studies found correlations between OCP levels in maternal blood and the placenta, the association between low levels of OCPs in serum of pregnant women and fetal NTD risk remains unknown. Therefore, the aim of this study was to examine the association between OCP concentrations in maternal serum and risks of NTDs in offspring.

2. Material and methods

2.1 Study population

Subjects were recruited from Taigu, Pingding, Xiyang, Shouyang, Zezhou, and Changzhi in Shanxi Province, China from November, 2010 to March, 2013. This area was selected because it has a relatively higher NTD rate and higher levels of OCP residues in the placenta than other sites (Ren et al., 2011). NTD cases were determined through a population-based birth defects surveillance program which showed an overall NTD prevalence of 13.9 per 1000 pregnancies (Li et al., 2006). In the surveillance program, major external structural birth defects, including NTDs, were ascertained by active case monitoring. The control group consisted of mothers who delivered healthy infants with no congenital malformations. For every NTD case, the mother of a healthy newborn was selected as a control at the same hospital or clinic. The controls were selected to match cases by mother's place of residence and last menstrual period (± 2 weeks). Information on mother's socio-demographic characteristics, reproductive history, periconceptional folate supplementation, fever or flu during early pregnancy, drinking alcohol, active or secondhand smoking during the periconceptional period was collected by face-to-face interviews conducted by trained local health workers at hospitals and clinics within the first week of delivery or pregnancy termination. We took the following measures to minimize the potential information bias. First, we trained local health workers at hospitals and clinics to standardize the interview procedures; second, the structured questionnaire contained questions that are straightforward. We did an independent survey on the accuracy and reproducibility of the case-control data, and found the coincidence rate for major variables collected in the case-control study was 93.4% (Li et al., 2011). Fasting blood samples of 117 NTD case mothers (anencephaly, 44; spinal bifida, 67; encephalocele, 6) and 121 control mothers were collected at delivery or termination of NTD-affected pregnancies. Serum was separated immediately, stored at -20°C , and transferred on dry ice to the laboratory for processing and analyses. All the serum samples were kept at -80°C until extraction. The study protocol was approved by the institutional review board of Peking University, and written consent from the mothers was obtained.

2.2 Solvents and reagents

Acetonitrile and *n*-hexane of Ultra Resi-Analyzed[®] were purchased from Merck, Germany, and acetone and dichloromethane of Suprasolv[®] from J.T. Baker[®], USA. Silica gel (100–200 mesh), neutral aluminum oxide (200–300 mesh), and granular anhydrous sodium sulfate were all from Beijing Reagent, China. Silica gel and neutral aluminum oxide were heated at 450°C for 4 h, and the anhydrous sodium sulfate was baked at 600°C for 6 h to remove impurities. Silica gel was further reactivated, then kept in a sealed desiccator and heated at 130°C for at least 16 h before use. Granular anhydrous sodium sulfate was stored in a sealed glass bottle after cleaning. All glassware was cleaned with an ultrasonic cleaner (KQ-500B, Kunshan, China) for 30 min with liquid detergent, rinsed with distilled water, and afterward baked at 400°C for 6 h.

2.3 Laboratory Analyses

The extraction and cleanup procedures for OCP analysis have been described previously (Yu et al., 2013). A modified method of OCP extraction using microwave extraction method was described in (Wang et al., 2011). Briefly, 0.6–1.5 ml serum spiked with the recovery surrogate (1-bromo-2-nitrobenzene, J&K Chemical, USA) was extracted with 20 ml acetonitrile using microwave extraction system (CEM Mars Xpress, USA, 1200W). The temperature program was as follows: increasing from room temperature to 110°C in 10 min and holding at 110°C for another 10 min. The lipid in the extract was filtered using a 37 mm (diameter) glass fiber filter (0.22 µm). The filtrate was transferred to a 250-ml separatory funnel and then extracted with 30 ml *n*-hexane with a 4% sodium sulfate solution, twice. The extraction (about 60 ml *n*-hexane) was concentrated to about 1 ml in a rotary evaporator and transferred to a silica/alumina gel cleanup column (30 cm×10 mm i.d.) containing from bottom to top: 12 cm alumina, 12 cm silica gel, and 1 cm anhydrous sodium sulfate; and conditioned with 10 ml hexane). The column was eluted with 50 ml hexane/dichloromethane (1:1, v/v), and the eluate was concentrated to about 1 ml and spiked with an internal standard (pentachloronitrobenzene, from J&K Chemical, USA). A gas chromatograph (GC, Agilent 7890) coupled with a mass spectrometer (MS, Agilent 5975) and equipped with a HP-5MS capillary column (30 m × 0.25 mm × 0.25 µm) was used to detect OCPs. The GC oven temperature program was 50°C for 2 min, increased to 150°C at a rate of 10°C/min, to 240 at 3°C/min, and to 300°C, and held for 58 min. Helium was used as the carrier gas. OCPs were identified based on the retention times and qualifying ions of standards in selected ion monitoring mode with negative chemical ionization source. The 25 OCPs (OCP mix standards, from J&K Chemical, USA) including pentachlorobenzene (PeHB), hexachlorobenzene (HCB), *α*-hexachlorocyclohexane (*α*-HCH), *β*-hexachlorocyclohexane (*β*-HCH), *γ*-hexachlorocyclohexane (*γ*-HCH), *δ*-hexachlorocyclohexane (*δ*-HCH), *o,p'*-dichlorodiphenyltrichloroethane (*o,p'*-DDT), *p,p'*-dichlorodiphenyltrichloroethane (*p,p'*-DDT), *o,p'*-dichlorodiphenyldichloroethylene (*o,p'*-DDE), *p,p'*-dichlorodiphenyldichloroethylene (*p,p'*-DDE), *o,p'*-Dichlorodiphenyldichloroethane (*o,p'*-DDD), *p,p'*-Dichlorodiphenyldichloroethane (*p,p'*-DDD), heptachlor, aldrin, isodrin, heptachlor epoxide (A) (Hepta-EpoA), heptachlor epoxide (B) (Hepta-EpoB), oxychlordane, *α*-chlordane, *γ*-chlordane, endosulfan, dieldrin, endrin, endosulfan I, endosulfan II, methoxychlor and mirex were measured.

Triglycerides and cholesterol contents were measured by the oxidase method (INTEC (Xiamen) Technology Co., LTD, China) following the standard protocol. The optical density was quantified by an automatic biochemical analyzer (Olympus AU400, Japan).

2.4 QA/QC

Two procedure blanks and a reagent blank were included for each batch (18–20 samples) of serum samples. The possible degradation of *p, p'*-DDT in the injector was routinely checked. The recoveries of the surrogate standard in serum samples varied from 22.8% to 139% with the mean and standard deviation ($73.4 \pm 18.7\%$). To evaluate the extraction efficiency of the OCPs analysis method, a serum sample was divided in 6 aliquots and 25 OCPs were spiked on three of them. Recoveries for the 25 OCP standards varied from 56.3% (δ -HCH) to 117% (β -HCH) with the mean and standard deviation ($94.3 \pm 14.7\%$). The detailed recoveries for them were shown in Figure 1 (Appendices).

2.5 Data analyses

The study was originally designed as a matched case-control study. However, some blood samples were not available for evaluation because consent could not be obtained from some women, or collecting a blood sample from a control mother matched to a terminated case mother was often delayed because we had to wait till term delivery of the control mother. To maximize the sample size, we broke the pairs in data analyses. We examined the distribution of the two matching variables in the two groups and found no difference.

The OCP concentration in serum was normalized by lipid content and reported as ng/g lipid. Total lipid content (*TL*, g/l) was calculated based on the concentrations of triglycerides (*Tg*, g/l) and total cholesterol (*Tc*, g/l) using the formula $TL = 0.92 + 1.31 \times (Tg + Tc)$ (Rylander et al., 2006). The limits of detection (LOD) of all chlorinated compounds in serum ranged from 0.03 to 0.10 ng/mL. Samples with concentrations below the LOD were assigned to be zero. Because the measured OCP concentrations were not normally distributed, the median with the interquartile range (IQR) was used to describe the skewed distributions of the chemical concentrations. Difference in proportions between groups was examined with Pearson's χ^2 test. Non-parametric analysis was used to compare the medians between the case and control groups. For dose-response analysis, concentration quartiles of total OCPs of the control group were used as the cutoff values. The risk of NTDs associated with OCP levels was estimated by odds ratio (OR) with 95% confidence interval (CI). Binary unconditional logistic regression was used for the trend analysis. We took NTDs status as dependent variable, level of OCPs exposure (in quartile category) as independent variable, whilst adjusting for the factors that were significantly different between the case and the control group: maternal occupation, age, educational level, prepregnancy body mass index (BMI), periconceptional folic acid supplementation, active or secondhand smoking and drinking alcohol during the periconceptional period, and fever or flu during early pregnancy. Statistical analyses were conducted using SPSS 16.0. A two-tail *p* value of < 0.05 was considered to indicate statistical significance.

3. Results and Discussion

3.1 Characteristics

Characteristics of the subjects participating in this study and the associated univariate risk for NTDs were summarized in Table 1. There was a significant difference between the two groups with respect to maternal age, prepregnancy BMI, education, periconceptional folate supplementation, fever or flu occurrence and alcohol drinking during early pregnancy. There tended to be slight differences for maternal occupation ($p = 0.057$) and previous birth defects history ($p = 0.066$).

It has previously been known that BMI is a potential risk factor for NTDs while periconceptional folate supplementation acts as a protective measure (Copp et al., 2013). Gao and collaborators (2013) reported that maternal obesity before pregnancy was associated with the risk of NTDs (Gao et al., 2013). Periconceptional folate supplementation has been proved to be an effective method to reduce NTD risk (Copp et al., 2013). However, the association between periconceptional folic acid supplementation and the reduced NTDs risk was weaker in overweight/obese mothers (BMI ≥ 24.0 kg/m²) than in underweight/normal weight mothers (BMI < 24.0 kg/m²) (Wang et al., 2013b). These studies concluded that maternal obesity counseling should be considered in pre-conception care. In this study, the differences of maternal smoking and drinking alcohol during the periconceptional period between the two groups indicated that life style may play an important role in fetal NTD risk in offspring. We did not find maternal occupation (farmer vs. non-farmer) to be associated with NTD risk. This is not consistent with a previous report, which indicated that women as farmers were at a higher risk of carrying a fetus affected with an NTD (Fear et al., 2007). Overall, most of the above characteristics had a significant relationship with either elevated or decreased fetal NTD risks and should be considered as confounders in the evaluation of the association between maternal OCP concentration and the risk of NTDs.

3.2 Serum OCP Concentration

Among the 25 measured OCPs, seven of them were detected in more than 5% of the total samples, including PeCB, HCB, α -HCH, β -HCH, γ -HCH, p,p' -DDE and p,p' -DDT. Their concentrations and detection rates along with comparisons with other previous studies are shown in Table 2. Among the measured HCH isomers, α -HCH was detected in 53% of the total 238 samples. This finding is different from other studies (Kang et al., 2008; Karami-Mohajeri and Abdollahi, 2011; Wang et al., 2013a), in which β -HCH had the highest detection rate and concentrations. This discrepancy cannot be explained with the data obtained in this study. p,p' -DDE, a metabolite of p,p' -DDT, had the second highest detection rate. Its concentration and detection rate were higher than those of p,p' -DDT and other DDT-like compounds, which is consistent with studies conducted in other countries (Ali et al., 2013; Jakszyn et al., 2009; Waliszewski et al., 2012; Zhou et al., 2013).

Except for the HCB, the serum concentrations and detection rates of OCPs were lower than those reported in other cities like Guangzhou (Qu et al., 2010), Shanghai (Cao et al., 2011), and Hong Kong (Wang et al., 2013a), and other countries in Asia, Europe and North America (Ali et al., 2013; Amodio et al., 2012; Dirtu et al., 2006; Itoh et al., 2009; Jakszyn

et al., 2009; Kalantzi et al., 2011; Kang et al., 2008; Thomas et al., 2006; Waliszewski et al., 2012) as shown in Table 2. It can be noted that the two studies conducted in Mexico and Spain (Jakszyn et al., 2009; Waliszewski et al., 2012) reported significant higher OCP concentration than our study. This higher concentrations could result from using electron-capture detection (ECD), which may cause serious false positive results (Pyle et al., 1998). The lower concentrations observed in this study may be explained by the fact that most of the subjects were recruited from a rural area with little cotton farming and lower socioeconomic development. Compared with urban population, they had less consumption of foods prone to accumulate organic contaminants, such as meat, eggs, milk, and seafood, which could add to the effect resulting from exposure to OCPs (Wang et al., 2013a; Yu et al., 2013).

3.3 Comparison of OCP Concentration between Case and Control Groups

Overall, the serum concentrations of HCB and total OCPs (Σ OCPs) of the case group were higher than those of the control group, while there were no significant differences for the other detected OCPs (α -HCH and p,p' -DDE) as shown in Table 3. For p,p' -DDE, its median concentration (64.2 ng/g lipid) was higher in the case group than that in the control group (15.7 ng/g lipid), but the difference was not statistically significant ($p = 0.063$). For the three subtypes of NTD cases, only the concentrations of HCB in anencephaly group were significantly higher than those in the control group. For anencephaly and spina bifida, their Σ OCP median concentration (118 and 125 ng/g lipid, respectively) was marginally higher than that of the control group (93 ng/g lipid), ($p = 0.091$ and 0.086 , respectively). As only 6 subjects with encephalocele were recruited, this NTD subtype will not be discussed in the following sections. The Σ OCP concentrations in subgroups of potential confounders were listed in Table 1 (Appendices), including maternal occupation, age, educational level, prepregnancy BMI, periconceptional folic acid supplementation, active or secondhand smoking and drinking alcohol during the periconceptional period, and fever or flu during early pregnancy. The Σ OCP concentrations in case group were found to be higher than the control group in subgroups of maternal age between 25 – 29 years, BMI ≥ 25 kg/m², farmer, previous birth defect history, and periconceptional folate supplementation, while for the other subgroups, no significant differences were found.

3.4 Association between Serum OCPs and NTD risk

No apparent dose-response relationship between serum levels of Σ OCPs and the risk of NTDs was found for both Σ NTDs and the two major subtypes in either unadjusted or adjusted model (Table 4). In the unadjusted model, when the lowest quartile (59.9 ng/g lipid) was used as reference, a marginally significant increasing trend ($p = 0.054$) in the risk of NTDs of 0.826-fold (95% CI, 0.375–1.82), 1.47-fold (95% CI, 0.712–3.05), and 1.74-fold (95% CI, 0.846–3.58) were observed for women whose serum concentration of Σ OCPs were in the second, third, and forth quartiles, respectively. No consistent trend was observed in the adjusted model for anencephaly and spina bifida subtypes. The NTD risks in association with the six individual OCPs were listed in Table 2 (Appendices) and no significant elevated NTD risk was observed.

These results were not completely consistent with our previous study using placental samples which showed a clear dose-response relationship between higher levels of total *o,p'*-DDT and α -HCH with the risk of NTDs (Ren et al., 2011). Since the maternal serum samples of the present study and the placental samples of our previous study (Ren et al., 2011) were not collected from the same individuals, we were unable to test whether there was a positive correlation between the OCP concentrations in the two types of biological samples, as was observed in the studies by Bergonzi et al. (2009) and Yu et al. (2013). OCPs, especially HCH and DDT, have been proved to be capable of inducing reactive oxygen species and further oxidative stress damage (Hansen, 2006). They could cause adverse biological effects and could result in reproductive and developmental damage even at low levels in the human body (Androutsopoulos et al., 2013). However, no significant elevated NTD risk associated with serum OCP residues was found in the present study.

Although more subjects (117 cases and 121 controls) were included in this study when compared with our previous one (80 cases and 50 controls) (Ren et al., 2011), the detection rate of OCPs in sera in the present study was lower than that in placental tissue in the previous study. This might limit the power to detect a significance in dose-response analysis.

4. Conclusions

Although the overall OCP concentration in serum of women who had pregnancies affected by NTDs was higher than those who delivered healthy infants, no dose-response relationship between higher levels of any individual OCP or total OCPs and the risk of total NTDs or subtype was found in either the unadjusted model or the model adjusted for confounders. Therefore, no clear association between maternal serum OCP residues and the risk of NTDs in offspring was observed in this population. As this is the first report for OCP comparison between NTD case mothers and control mothers to our knowledge, further studies with larger sample size is needed to replicate the findings in other populations and to examine whether maternal serum OCP residues is an appropriate biomarker of NTD risk in offspring.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- The association between maternal serum OCP concentrations and NTD risk was investigated in a case-control study
- The total OCP concentration of the NTD group was significantly higher than that of the control group
- No dose-response relationship between higher levels of any individual OCP or total OCPs and the risk of NTDs was found
- No clear association between maternal serum OCP residues and NTD risk was observed

Table 1

Characteristics of women who had pregnancies affected by NTDs (cases) and women who delivered healthy infants (controls) and their associated univariate risk for NTDs.

Characteristics	Cases (<i>n</i> = 117) [†]	Controls (<i>n</i> = 121) [†]	<i>p</i> [‡]	Unadjusted OR [§] (95% CI)
Maternal age(y)				
< 25	45 (40)	69 (57)		1.0
25 – 29	23 (29)	31 (26)	0.011	1.52 (0.82 – 2.81)
30	35 (31)	20 (17)		2.14 (1.11 – 4.12) *
Maternal prepregnancy BMI (kg/m ²)				
< 18.5	12 (11)	12 (10)		1.0
18.5–24.9	60 (54)	82 (70)	0.032	0.840 (0.352 – 2.00)
25	38 (35)	23 (20)		2.61 (0.992 – 6.88)
Maternal education				
Primary or lower	9 (8)	2 (2)		1.0
Junior high	85 (73)	67 (55)	< 0.001	0.267 (0.056 – 1.28)
High school or above	22 (19)	52 (43)		0.107 (0.021 – 0.532) **
Maternal occupation				
Farmer	93 (80)	84 (69)	0.057	1.0
Non-farmer	23 (20)	37 (31)		0.616 (0.340 – 1.12)
Previous birth defects history				
No	108 (95)	114 (99)	0.066	1.0
Yes	6 (5)	1 (1)		6.11 (0.724 – 51.58)
Periconceptional folate supplementation				
No	64 (55)	48 (42)	0.041	1.0
Yes	52 (45)	67 (58)		0.770 (0.459 – 1.29)
Fever or flu during early pregnancy				
No	52 (46)	88 (76)	<0.001	1.0
Yes	62 (54)	28 (24)		3.19 (1.83 – 5.55) ***
Maternal smoking or secondhand smoking				
No	37 (33)	84 (74)	<0.001	1.0
Yes	74 (67)	30 (26)		3.99 (2.29 – 6.96) ***
Alcohol drinking				
No	101 (86)	113 (93)	<0.001	1.0
Yes	16 (14)	8 (7)		2.77 (1.10 – 6.95) *

[†]Data are number (percentage). Total number may not be equal to the number of cases or controls due to missing or unknown data.

[‡]Pearson's χ^2 test, or Fisher's exact test if cell expectation was less than 5.

* $p < 0.05$,

** $p < 0.01$,

*** $p < 0.001$ in comparison with the reference group, using binary unconditional logistic regression

Table 2

Comparison of serum OCP median concentration and detection rate above the limit of detection between this study and the previous reported studies.

Country (City or region)	Sample year	Gender [‡]	Sample number	Chemical detector [*]	OCP median concentration and detection rate in serum (ng/g lipid)							References
					PeCB	HCB	α -HCH	β -HCH	γ -HCH	p,p' -DDE	p,p' -DDT	
China (Shanxi)	2010–2012	F		GC-MS (NCI)	0 (32) [‡]	42 (92)	5.2 (53)	0 (37)	0 (20)	64 (82)	0 (5.9)	This study
China (Guangzhou)	2004	F	30	GC-MS	n.r. [§]	n.r.	3.9	35	2.9	1600	120	Qu et al., 2010
China (Hongkong)	2011	F	54	GC-MS	n.r.	n.r.	18 (96)	221 (100)	151 (100)	224 (96)	75 (96)	Wang et al., 2013a
Spain	1993–1994	F	479	GC-ECD	n.r.	377 (78)	n.r.	n.r.	194 (87)	789 (99)	0.0 (27)	Jakszyn et al., 2009
	2001–2005	F (Breast Cancer case)	403		n.r.	27 (100)	n.r.	n.r.	n.r.	360 (100)	9.3 (100)	
Japan (Nagano Prefecture)	2001–2005	F (Breast Cancer Control)	403	GC-Isotope MS	n.r.	27 (100)	n.r.	n.r.	n.r.	370 (100)	9.9 (100)	Itoh et al., 2009
Greece (Attika)	2007	F	61	GC-MS (EI)	n.r.	23 (100)	0.02 (2)	18 (100)	n.r.	268 (100)	6.3 (100)	Kalanzi et al., 2011
Mexico (Veracruz)	2010	F	75	GC-ECD	n.r.	n.r.	n.r.	2200 (57)	n.r.	7800 (100) [‡]	2200 (51)	Waliszewski et al., 2012
Pakistan (Islamabad)	2012	F (Rural)	17	GC-MS (NCI)	n.r.	3.6 (100)	n.r.	n.r.	n.r.	142 (94)	5.5 (94)	Ali et al., 2013
	2012	F (Urban)	17		n.r.	14 (100)	n.r.	n.r.	n.r.	155 (100)	7.5 (71)	
United Kingdom	2003	F&M	154	GC-MS (EI)	n.r.	11 (70)	0 (0)	n.r.	0 (0)	100 (100)	2.9 (91)	Thomas et al., 2006
Romania (Iassy)	2005	F&M	142	GC-MS (NCI)	n.r.	30 (99)	31 (100)	923 (100)	127 (98)	1975 (100)	93 (100)	Dirtu et al., 2006
Republic of Korea (Seoul)	2006	F&M	40	GC-HRMS (EI)	n.r.	n.r.	0 (2.5)	49 (100)	0 (2.5)	224 (100)	19 (73)	Kang et al., 2008
Italy (Western Sicily)	2009	F&M	101	GC-MS (EI)	n.r.	19	4.1	4.3	1.4	175	4.4	Amodio et al., 2012

[‡]F stands for female, M for male, F&M for female and male.

[‡]Data are median (detection rate, %). The median “0” stands for “neidan is below detection limit”.

[§]Data are not reported.

^{*} GC stands for gas chromatography, MS for mass spectrometer, NCI stands for negative chemical ionization or electron capture negative ionization, ECD for electron-capture detection, EI for electron impact ionization

Concentrations (ng/g lipid) of detected OCPs with detection rate more than 50% in the serum samples of women who had pregnancies affected by NTDs (cases) and women who delivered healthy infants (controls)

Table 3

OCPs	Control (n ^f = 121)		ΣNTDs (n = 117)		p [‡]	Anencephaly (n = 44)		p [‡]	Spina bifida (n = 67)		p [‡]	Encephalocele (n = 6)		p [‡]
	% [‡]	Median (IQR)	% [‡]	Median (IQR)		%	Median (IQR)		%	Median (IQR)		%	Median (IQR)	
HCB	93.4	25.0 (35.4–49.2)	91.5	42.1 (29.1–63.8)*	0.020	93.2	40.8 (29.5–62.7)*	0.048	89.6	42.5 (27.9–57.7)	0.125	100	52.1 (37.1–64.8)	0.097
α-HCH	55.4	7.54 (0.00–79.0)	51.3	5.17 (0.00–22.7)	0.658	56.8	8.84 (0.00–21.6)	0.527	44.8	0.00 (0.00–22.0)	0.804	83.3	19.7 (10.9–28.3)	0.092
p,p'-DDE	81.0	15.7 (45.4–94.6)	84.6	64.2 (27.3–130)	0.063	81.8	62.3 (24.7–157)	0.206	85.1	64.2 (27.2–127)	0.098	85.1	64.9 (39.6–70.8)	0.561
ΣOCPs [§]		93.0 (54.8–170)		120 (66.1–230)*	0.035		118 (60.1–278)	0.107		125 (72.1–200)	0.110		128 (103–263)	0.215

IQR, Interquartile range.

^fNumber of subjects.

[‡]Detection rate of a specific OCP in serum samples.

[‡]In comparison with the controls, Mann-Whitney U test.

* p 0.05 in comparison with the median of controls, Mann-Whitney U test.

[§]Including HCB, α-HCH, and p,p'-DDE with detection rate above 50%

Risks of NTDs and its two major subtypes with the four total serum concentration (Σ OCPs) groups of the control with the corresponding number of samples (30, 30, 31, and 30)

Table 4

Group [£]	No. of subjects	Unadjusted [†]		Adjusted [‡]	
		OR [§] (95% CI)	<i>P</i> _{trend} [§]	OR (95% CI)	<i>P</i> _{trend}
ΣNTDs [*] (<i>n</i> =117)					
G1	23	1.00		1.00	
G2	19	0.826 (0.375–1.82)		0.451 (0.167–1.223)	
G3	35	1.47 (0.712–3.05)	0.054	1.243 (0.504–3.067)	0.199
G4	40	1.74 (0.846–3.58)		1.342 (0.539–3.345)	
Anencephaly (<i>n</i> =44)					
G1	9	1.00		1.00	
G2	6	0.187 (0.580–1.80)		0.328 (0.083–1.30)	
G3	14	1.35 (0.519–3.53)	0.340	1.05 (0.314–3.50)	0.316
G4	15	1.27 (0.488–3.30)		1.31 (0.409–4.21)	
Spina bifida (<i>n</i> =67)					
G1	14	1.00		1.00	
G2	12	0.829 (0.329–2.09)		0.401 (0.118–1.36)	
G3	18	1.31 (0.556–3.10)	0.259	0.847 (0.275–2.61)	0.774
G4	23	1.42 (0.616–3.29)		0.892 (0.290–2.74)	

[§] Odds ratio

* Number of subjects

^f Σ OCPs concentration groups were divided into 4 groups based on the quartiles of total OCPs of the control group, which are G1 (< 59.9), G2 (59.9 – 111), G3 (111 – 196), and G4 (> 196) ng/g lipid, respectively. Σ OCPs was including HCB, α -HCH, and *p,p'*-DDE

^g In comparison with controls; exposure was defined as above the median concentration.

^h Adjusted for maternal occupation, age, educational level, BMI, folic acid supplementation, active or secondhand smoking and drinking alcohol during the periconceptional period, fever or flu during early pregnancy.

[§] *p*_{trend} is calculated by using binary logistic regression